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Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular endopeptidases that have traditionally been viewed as effectors of late stages of cancer evolution. Recently, MMP-3 and MMP-14 were shown to act as natural tumor promoters when overproduced in mouse mammary glands (Sternlicht et al., 1999; Ha et al., 2001). To understand how these MMPs can alter cell behavior and influence breast cancer susceptibility, the yeast two-hybrid system was used to identify interacting proteins as potential novel MMP substrates. Three different 'bait' constructs were generated for each mouse and human MMP, but only the hemopexin domain hybrids appear suitable for screening. The catalytic domain hybrids are 'sticky' and auto-activate one or both reporters. The full-length hybrids also contain this 'sticky' catalytic domain and are probably poorly folded, as mature MMPs expressed in the yeast cytoplasm are inactive. Screening of a mouse and human mammary gland cDNA library from ATCC identified to date only intracellular interacting proteins. As the mRNA sources for these libraries were less than optimal, we may look for other libraries or make our own. In the latter case rough-ER bound mRNA will be isolated, to enrich for clones encoding membrane and secreted proteins.

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## Introduction

The MMPs constitute a family of extracellular proteolytic enzymes that participate in both physiological and pathological processes. Their contribution to cancer development was initially thought to be restricted to progression, due to their positive influence on angiogenesis required for the growth of solid tumors and their potent ability to degrade structural extracellular matrix (ECM) components that normally prevent invasion and metastasis. However, Sternlicht *et al.* (1999) and Ha *et al.* (2001) demonstrated that overproduction of Stromelysin-1 (MMP-3) or MT1-MMP (MMP-14) in mouse mammary glands is sufficient to induce hyperplasias and breast cancer. The *in vitro* substrates of these MMPs include most if not all structural ECM proteins and a growing list of cell-surface molecules, but their *in vivo* substrates and the actual mechanism underlying their ability to modify cell behavior and induce carcinogenesis remains poorly understood. This project aims to identify novel interacting proteins for MMP-3 (Stromelysin-1) and MT1-MMP (MMP-14) by yeast two-hybrid screening, demonstrate their cleavage *in vitro* and subsequently assess their tumor promoting ability *in vivo*. Here I report my findings for the first year of this postdoctoral traineeship award.

## Body

*Task 1a + 1b.* Stromelysin-1 and MT1-MMP have a domain architecture common to most MMPs: 1) a N-terminal signal sequence that is removed upon entry in the endoplasmic reticulum, followed by 2) a propeptide that maintains enzyme latency until it is removed, 3) a catalytic domain that contains a highly conserved zinc-binding motif and 4) a hemopexin-like domain that is connected to the catalytic domain via a hinge region/linker. Stromelysin-1 is secreted into the ECM as a soluble proenzyme, while MT1-MMP is retained at the cell surface via a C-terminal transmembrane domain. In an attempt to mimic the *in vivo* MMP-substrate interaction as closely as possible, mature inactive Stromelysin-1 and MT1-MMP from both human and mouse was generated as 'bait'. This involved removal of the signal sequence, propeptide and C-terminal transmembrane domain (MT1-MMP only). Furthermore, an E to A substitution was introduced in the zinc-binding motif (HEBXHXBGBXHS: bold residues are invariant, X is a variable residue and B is a bulky hydrophobic residue) by site-directed mutagenesis to inactivate the MMPs (Stöcker *et al.*, 1995). In addition, the catalytic domain as well as the hemopexin domain with the hinge region of each MMP were also generated as 'bait', to help identify the domain involved in the MMP-protein interaction. The absence of point mutations and in frame fusion of these cDNA fragments to the DNA-binding domain of the *E. coli* lexA protein were confirmed by DNA sequencing. These constructs were introduced into the yeast two-hybrid strain Y1003 and expression of the different fusion proteins was confirmed by Western analysis using a commercially available antibody directed against the DNA-binding domain of lexA (data not shown). All 'baits' were tested for their ability to activate the reporter genes by themselves, in the presence of an empty library plasmid or in the presence of a protein known to interact with these MMPs (Table 1). Tissue inhibitor of metalloproteinases 1 (TIMP1) is known to bind Stromelysin-1 and TIMP2 is known to bind MT1-MMP (Woessner and Nagase, 2000).

Mature mouse MMP-3 is 83% identical at the amino acid level to its human ortholog, while mouse and human MMP-14 share 97% identity. It is therefore not surprising that the mouse MMP baits behaved in a manner that was indistinguishable from their human counterparts. The catalytic domain of MMP-14 activated both reporter genes even in the absence of a library plasmid and was therefore not used to screen for interacting proteins. The catalytic domain of MMP-3 is somewhat less 'sticky', as it activated only the lacZ reporter in the presence of a library plasmid. The catalytic domain of MMP-3 was included in the first round of screening, since adenine auxotrophy is used for the actual two-hybrid screen. Interestingly, the catalytic domain of MMP-3 did not interact with TIMP1 but did bind TIMP2, suggesting that these two TIMPs may inhibit MMPs through distinct mechanisms. It was previously shown that the MMP-inhibiting activity of TIMP1 does not tolerate additional amino acids at its N-terminus (Woessner and Nagase, 2000). It is therefore likely that the N-terminal fusion to the activation domain of Gal4p interfered with the TIMP1-MMP interaction. Unfortunately, neither the full length nor the hemopexin domain of both MMP-3 and MMP-14 interacted with TIMP1 or TIMP2. Thus at this stage it was still unclear whether these fusion proteins were able to enter the nucleus.

Problems encountered: 1) Multiple point mutations were present in the mouse MMP-14 clone, causing several amino acid substitutions at highly conserved positions which therefore had to be fixed. 2) The different 'bait' constructs initially displayed inconsistent interaction with either the empty library plasmid or the positive control TIMP constructs, due to a reversion of one of the two *ade2* loci by the two-hybrid *ADE2* reporter in a subpopulation of the host strain. This problem was solved by selecting for single colonies on synthetic media (YNB) that were unable to grow in the absence of adenine.

To further explore the feasibility of using mature inactive MMPs as 'bait', an additional experiment was performed to test whether these enzymes are active and thus properly folded in the yeast cytoplasm. To this extent the signal sequence and propeptide of mouse Stromelysin-1 were replaced with a starting methionine and the resulting active MMP-3 was subcloned in a yeast expression vector with an inducible promoter, since the expression of an active protease within the yeast cytosol was expected to be toxic. Surprisingly expression of active MMP-3 did not result in a detectable phenotype. In addition, cell lysates from these transformants did not display proteolytic activity towards the well-established MMP-3 substrate  $\alpha$ -casein (data not shown). Unfortunately, I was unable to definitively confirm MMP-3 expression by Western analysis due to a lack of a specific antibody. However, similar results were obtained with mouse MMP-2, human MMP-9 and mouse MMP-13 and expression of the latter two was confirmed by Western analysis. Collectively, these observations suggest that full length MMPs are poorly folded in the reducing environment of the yeast cytoplasm and may therefore be less suitable as 'bait' in a yeast two-hybrid screen.

*Task 1c.* A mouse and human mammary gland Gal4-AD cDNA library were obtained from the American Type Culture Collection (ATCC # 87294 and 87295) as phage lysates. These libraries were titrated and subsequently exposed to the bacterial strain BNN132 to convert them into plasmid DNA as described by Elledge *et al.* (1991).

The library plasmid pACT2 is maintained as a low copy plasmid in *E. coli* ( $\pm 15$  copies per cell), which required the growth of large culture volumes in order to obtain sufficient amounts of plasmid DNA. Approximately 11 mg of plasmid DNA was purified from 27 liter of *E. coli* culture, representing 272% coverage of the  $1 \times 10^8$  independent clones estimated to be present in the mouse cDNA library. The titer of the human library was significantly lower ( $7.5 \times 10^5$  pfu/ $\mu$ l versus  $2.8 \times 10^6$  pfu/ $\mu$ l for the mouse library) and only 4 mg of plasmid DNA was purified from 9 liter of *E. coli* culture, representing 85% coverage of  $1 \times 10^8$  independent clones estimated to be present in the human cDNA library.

*Task 1d.* The two-hybrid screen was started with MMP-3 domain 'baits' for the following reasons: a) the MMP-14 catalytic domains auto-activated both reporters and could not be used for screening, b) the mouse MMP-14 clone had several point mutations that needed to be fixed first, and most importantly c) our lab has direct access to MMP-3<sup>-/-</sup> animals as well as WAP-Stromelysin 1 transgenics. These animal models will almost certainly help with the *in vivo* validation of substrate cleavage of any interacting protein identified in this screen. The catalytic domain of mouse MMP-3 interacts with roughly 1 out of every 500 clones, while its human counterpart binds approximately 1 out of every 1000 clones. Sequence analysis of 10 random clones identified only intracellular proteins. Thus it appears that the MMP-3 catalytic domain is also a 'sticky' bait and cannot be used to enrich for potential extracellular substrates. The full-length MMP-3 baits were also used to screen, despite the fact that they are likely poorly folded. Approximately  $7.5 \times 10^5$  transformants were screened against human full-length MMP-3 and 326 candidates displayed activation of both reporters (1 out of  $\pm 2300$  clones). Although this result indicated that the full-length MMP-3 bait was able to enter the nucleus, it was decided that there were too many candidates to pursue. Roughly  $9.8 \times 10^5$  transformants were screened against mouse full-length MMP-3, of which 69 activated both reporters (1 out of  $\pm 14,000$  clones). After demonstration of plasmid dependent activation of both reporters, 57 'true' candidates remained. It was determined by PCR and restriction analysis that these plasmids contained 38 different cDNA inserts, which were further subdivided in three categories after sequence analysis: 1) sequence undetermined (2), 2) garbage (22) and 3) proper cDNA fusions (33). Table 2 depicts the unrelated 'garbage' activation domain hybrids that share certain amino acid repeats. It is inevitable that some out of frame fusions are identified that give rise to random peptides, but it is unusual to pick up this many clones with two amino acid repeats. These repeats were likely introduced during the construction of the library and are almost certainly the result of inappropriate ligation of multiple linkers to the 5' end of the cDNA. Table 3 lists the proper cDNA fusions that interact with full-length MMP-3. These 'true' positives were also tested directly against the catalytic domain and the hemopexin domain, but unfortunately all clones appear to encode intracellular proteins. Close to  $1.4 \times 10^6$  transformants were screened against the human MMP-3 hemopexin domain and 27 candidates were identified (1 out of  $\pm 52,000$  clones). Only five displayed plasmid dependent activation of both reporters and sequence analysis indicated that two different cDNA inserts were identified: 1) the same 78 aa fragment of an immunoglobulin lambda light chain VLJ region was picked up four times and 2) a 140 aa fragment of the bHLHZip transcription factor BIGMAXy. Finally,  $7.4 \times 10^5$  transformants were screened



against the mouse MMP-3 hemopexin domain and 120 candidates were identified (1 out of  $\pm 6200$  clones). Plasmid dependency was demonstrated for 98 candidates and I am currently in the process of sequencing these clones. I expect to have isolated both garbage clones as well as proper cDNA fusions that were previously identified with the full length MMP-3 bait, as several full length interactors appear to bind specifically to the hemopexin domain (see Table 2 and 3). The full length and hemopexin domain of MMP-14 have not yet been used in a two-hybrid screen.

Problems encountered: 1) Low initial transformation efficiency with both the mouse and human library. This issue was solved by growing the yeast strain in rich media for 4 hours prior to the transformation and by growing for 2 hours in 2H media after the transformation prior to plating. 2) Limited progress with the two-hybrid screen due to the unexpected high number of candidates that appear to specifically interact with the MMP baits. I am trying to design a PCR strategy to filter out the 'garbage' clones with two amino acid repeats. This would greatly reduce the number of plasmids that have to be isolated, retransformed and sequenced.

*Task 1e + 1f.* Thus far only intracellular interacting proteins have been identified, making further characterization of the MMP-protein interaction unnecessary.

### **Key Research Accomplishments**

- The catalytic domain of MMPs are 'sticky' and cannot be used in a yeast two-hybrid screen for interacting proteins as potential substrates
- Mature MMPs expressed within the yeast cytoplasm are not active and are therefore likely to be poorly folded
- Full length MMPs fused to the DNA-binding domain of *lexA* are able to enter the nucleus, but contain a 'sticky' catalytic domain and are poorly folded, making them less suitable as 'bait' in a yeast two-hybrid assay
- The hemopexin domain of MMPs contain substrate-binding 'exosites' and appear to be the best MMP domain to perform a yeast two-hybrid screen with

### **Reportable Outcomes**

None to date

### **Conclusions**

The observations presented here suggest that the hemopexin domain of Stromelysin-1 and MT1-MMP may represent the only domain of these MMPs suitable for use as 'bait' in a two-hybrid screen for interacting proteins as potential substrates. The catalytic domain of these MMPs is 'sticky' and did not appear to enrich for potential

extracellular substrates, while the full length MMPs are poorly folded due to a lack of glycosylation and/or disulfide bond formation in the yeast cytoplasm. This notion is further supported by the study of McQuibban *et al.* (2000), who identified the MMP-2 substrate MCP-3 in a two-hybrid screen with the hemopexin domain as bait.

Dr. Stephen J. Elledge is the depositor of both libraries and was contacted to obtain some details on their construction, since a relative large percentage of the interacting clones had two amino acid repeats and all the proper cDNA fusions only encoded intracellular proteins. A 10 week old virgin was the mRNA source of the mouse library, which is not optimal for this project as minimal deposition and/or remodeling of the ECM takes place at this developmental stage. Furthermore, the tissue used for the human library was derived from breast reduction mammoplasty, which is mostly fat pad and contains very little epithelial tissue. The mouse MMP-3 hemopexin screen will be finished, but it is likely that if no interesting interacting proteins are identified we may look for other mammary gland libraries or make our own. In the latter case rough-ER bound mRNA will be isolated to enrich for clones encoding membrane and secreted proteins (Diehn *et al.*, 2000).



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**Table 1. Interactions between the different 'bait' and 'prey' constructs**

	Gal4 only	mTIMP1	mTIMP2	hTIMP1	hTIMP2
lexA only	-	-	-	-	-
mM3-CD	-	-	++	nd	nd
mM3-HPX	-	-	-	nd	nd
mM3-FL	-	-	-	nd	nd
hM3-CD	-	nd	nd	-	++
hM3-HPX	-	nd	nd	-	-
hM3-FL	-	nd	nd	-	-
mM14-CD	++	++	+++	nd	nd
mM14-HPX	-	-	-	nd	nd
mM14-FL	-	-	-	nd	nd
hM14-CD	++	nd	nd	++	+++
hM14-HPX	-	nd	nd	-	-
hM14-FL	-	nd	nd	-	-

Abbreviations

lexA only: empty bait plasmid pEG202-NLS  
Gal4 only: empty library plasmid pACT2  
mM: murine MMP  
hM: human MMP  
CD: catalytic domain  
HPX: hinge region + hemopexin domain  
FL: full length

Two-hybrid interaction

+: weak  
++: intermediate  
+++: strong  
nd: not determined

**Table 2. Full-length mouse MMP-3 binds to unrelated proteins that share certain amino acid repeats**

	FL	CD	HPX
A) 9 clones with Glu-Arg and Phe repeats	++	++	++
B) 4 clones with only Glu-Arg repeats	++	++	-
C) 2 clones with only Phe repeats	+++	+	+++
D) 2 clones with Ser-Leu repeats	+	+++	-
E) 5 clones encode short peptides without similarity (out of frame fusions)			

**Table 3. Full-length mouse MMP-3 binds to intracellular proteins**

	Total number identified	Number of independent clones	FL	CD	HPX
A) Mitochondrial serine protease HTRA <sub>2</sub>	14	4	+++	+++	+
B) Unnamed protein containing valosin domain (transitional ER ATPase)	8	4	+++	+++	-
C) GADD 45 $\alpha$	3	1	+++	+++	-
D) Unnamed protein similar to GPS1	3	1	++	++	+
E) Endooligopeptidase A	3	1	++	+	+++
F) Unnamed protein similar to TRH <sub>4</sub>	1	1	++	+++	-
G) Leucineaminopeptidase	1	1	+	+	+++
H) HSP40 homolog DNAj	1	1	+	+	-